Granulocyte colony-stimulating factor (G-CSF) was reported to increase the number of colony-forming units-spleen (CFU-S) and multilineage colonies as well as myeloid-committed cells. We investigated the effects of G-CSF on myeloid progenitors and primitive stem cells in a mouse bone marrow transplantation (BMT) system. Lethally irradiated mice received BM cells from untreated or 5-fluorouracil-treated mice, and then were administered G-CSF or carrier buffer (control) for 5 days from immediately after BMT. A pre-CFU-S assay was performed by the repeated transplantation of BM cells from the first BMT recipients to other mice. By the method of polymerase chain reaction, most of the spleen colonies in the secondary recipients were confirmed to be derived from the first donors. G-CSF did not increase the peripheral white blood cell count significantly, but did increase the number of immature myeloid cells and granulocyte-macrophage colony-forming cells in the BM. The number of erythroid cells in the BM was initially suppressed and then increased by G-CSF treatment. In addition, the pre-CFU-S assay showed an increase in pre-CFU-S cells due to G-CSF administration. The number of spleen colonies of first BMT recipients did not increase, but a higher percentage of them were committed to a certain lineage by G-CSF treatment. These findings suggest that G-CSF has important roles in the early stages of hematopoiesis.

MATERIALS AND METHODS

Mice and 5-FU treatment. BDF, mice aged 8 to 11 weeks were purchased from Japan SLC (Shizuoka, Japan) and maintained under laminar air flow conditions with the acidified drinking water. To prepare BM treated with 5-FU (Hoffman La Roche, Basel, Switzerland), mice were injected intravenously with 5-FU (150 mg/kg) and then killed 4 days later. This 5-FU-treated BM (5-FUBM) or untreated normal BM was used as the first graft for BMT. From four to nine mice were examined for each set of experimental conditions.

Assay of spleen colonies. Spleen colonies or CFU-S were assayed by the intravenous injection of BM suspension (1 x 10^4 nucleated cells) on day 0 into irradiated (9.2 Gy) syngeneic BDF mice. On days 8 and 12, the nodules that developed on the spleen surface were counted after fixation. Irradiated mice without BM injection were confirmed to develop no spleen colonies.

G-CSF administration. Highly purified recombinant human G-CSF was provided by Kirin Brewery Co Ltd (Tokyo, Japan); the specific activity was 1 x 10^8 U/mg protein. G-CSF dissolved in phosphate-buffered saline (PBS) was injected subcutaneously once a day on days 0 to 4 (12 hours after BMT on day 0 and the other 4 days later. This 5-FU-treated BM (5-FUBM) or untreated normal BM was used as the first graft for BMT. From four to nine mice were examined for each set of experimental conditions.
injections at 24-hour intervals) after the first BMT at a dose of 2 μg/mouse/d. As a control, PBS alone was administered.

**Sequential BMT.** Repeated BMT was performed on day 12 after the first BMT. Femoral BM cells were collected from the recipients of the first BMT and 1 × 10^7 nucleated cells from normal BM recipients or 2 × 10^7 nucleated cells from 5-FUBM recipients were transplanted to each irradiated (9.2 Gy) second recipient mouse. After fixation, spleen colonies were counted and analyzed for their origin on days 8 and 12 after repeated BMT.

**Blood and BM examination.** Blood samples were drawn from the tail vein and the white blood cell count (WBC) was determined on days 6 and 11 after the first BMT. The femoral BM was incubated cell count (NCC), the differential count, and the assay of granulocyte-macrophage colony-forming cells (GM-CFC) were performed on days 8 and 12 after the first BMT. The WBC and NCC were determined using Türk’s solution and the counting plate. Differential counts were performed on cytocentrifuge preparations (obtained using a cytocentrifuge from Shandon Southern Instruments Inc, Sewickley, PA) stained with May-Grunwald-Giemsa solution.

**Assays of GM-CFC.** Quantification of GM-CFC was performed using a semisolid culture system as described previously. One milliliter of culture medium in a 35-mm dish contained 1 × 10^5 BM nucleated cells, 100 U/mL of murine recombinant interleukin-3 (IL-3; generously provided by Dr Ihle, National Cancer Institute, Frederick, MD), 2 U/mL of murine recombinant erythropoietin (Epo; generously provided by Chugai Pharmaceutical Company, Tokyo, Japan), 1.2% methylcellulose (Aldrich Chemical Co, Milwaukee, WI), 30% fetal calf serum (Flow Laboratories, North Ryde, Australia), 1% deionized bovine serum albumin (Sigma Chemical Co, St Louis, MO), 0.1 mmol/L mercaptoethanol (Sigma), and α-medium. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The colonies were counted by light microscopy 7 days after culture started, and two to four dishes were used to investigate each set of experimental conditions. GM-CFC-derived colonies (GM colonies) were identified morphologically, and in some cases the identification was confirmed by collecting the colonies and making cytocentrifuge preparations.

**Analysis of spleen colonies by polymerase chain reaction (PCR).** Spleens were fixed in 90% ethanol and 10% PBS. Then a small tissue fragment (about 0.3 mg) was enucleated from the center of each spleen colony. After washing by distilled water, the fragment was digested in 200 μL containing 500 μg/mL proteinase K (Boehringer Mannheim Biochemica, Mannheim, Germany), 50 mmol/L Tris-chloride (pH 8.5), 1 mmol/L EDTA, and 0.5% Tween 20 at 37°C overnight with shaking. Then the sample was incubated at 95°C for 10 minutes and 2 μL of it was subjected to PCR amplification (94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes for 40 cycles), using 2.5 units Ampli Taq (Perkin Elmer Cetus, Norwalk, CT), 100 ng each primer, and 100 μmol/L each dNTP (Boehringer) in a final reaction volume of 50 μL buffer recommended by the manufacturer (Cetus). For amplification of Y-chromosome-specific sequence (candidate for testis-determining gene), the sequence of the sense primer was 5’-GACTGGTG-GACAATTGTCTAG-3’ and the antisense primer sequence was 5’-TAAATGCCACTCCCTGTTG-3’. Amplification of β-actin sequence, the sense and antisense primer sequences were 5’-GTACACAGGCCATTTGTGATG-3’ and 5’-GCAACATAGCACAGCTTCTC-3’, respectively. Amplified products were electrophoresed on a 3% agarose and stained by ethidium bromide.

**Histologic analysis of spleen colonies.** Spleens were fixed in Bouin’s solution and embedded in paraffin, and sequential sections were prepared for light microscopic observation by conventional methods. Spleen colonies were classified morphologically into erythroid, granulocytic/monocytic, or mixed colonies. The total number of colonies per spleen determined by this method was almost the same as that determined by surface observation of the spleen. Two or three spleens were investigated for each set of experimental conditions.

## RESULTS

**Effects of G-CSF on the WBC and BM.** In both normal BM and 5-FUBM recipients, the peripheral WBC showed no significant difference between G-CSF–treated and control mice, but the femoral BM NCC and the GM-CFC were significantly increased in G-CSF–treated mice on days 8 and 12 (except for the NCC of 5-FUBM recipients on day 8). The GM-CFC increase in numbers was 1.5 to 17 times and the increase was larger than that of the NCC (Table 1). Differential counts of the BM showed that the increase in the NCC induced by G-CSF was mainly due to an increase in the number of myeloblasts and neutrophils. Interestingly, on day 8 the erythroblast count was larger in control mice than in G-CSF–treated mice, but on day 12 the reverse was true. These changes were observed in both normal BM and 5-FUBM recipients (Fig 1).

When the BM cells from day 8 5-FUBM recipients were cultured, the size of GM colonies showed an interesting pattern. Small GM colonies were predominant in G-CSF–treated mice, but large colonies were more common in control mice. The same tendency was also observed in the mice transplanted with untreated grafts, although it was not so distinct (Fig 2).

We followed several mice for a long time. At 7 weeks...
after BMT, there were no significant differences between G-CSF-treated and control mice in BM NCC, numbers of GM-CFC, and differential counts of the BM (data not shown).

**Assessment of pre–CFU-S.** The number of spleen colonies of 5-FUBM recipients without G-CSF treatment increased between day 8 and day 12 after the first BMT. In contrast, in the normal BM recipients the number was almost constant. We estimated the total number of CFU-S, which is designated the marrow repopulating ability (MRA) [CFU-S], in femoral BM specimens from the first BMT recipients on day 12 after the first BMT. The MRA[CFU-S] was determined as the number of spleen colonies that developed in second recipients multiplied by the femoral BM NCC of the first recipients on day 12. In mice without G-CSF treatment the MRA[CFU-S] values in 5-FUBM recipients were much larger than in normal BM recipients (Table 2). As reported previously, this implies that femoral BM CFU-S intrinsically migrated to the spleen and formed delayed colony there, and that sensitivity to 5-FU distinguishes CFU-S in the first BMT graft from CFU-S in the second BMT graft. Therefore, in this setting, CFU-S in the femoral BM of the first recipients are comparable with pre–CFU-S at the time of the first BMT, as Hodgson and Bradley mentioned. Thus, the MRA[CFU-S] is an appropriate index for indicating the total number of pre–CFU-S grafted to the first BMT recipient.

**Effects of G-CSF on spleen colonies and pre–CFU-S.** G-CSF treatment had no effect on the number of spleen colonies of the first recipients. In contrast, G-CSF treatment of normal BM or 5-FUBM recipients caused spleen colonies in the second recipients to increase from 2.0 to 5.6 times on both day 8 and day 12 after BMT. In addition, G-CSF increased the MRA[CFU-S] from 5.6 to 11.9 times in normal BM recipients and from 2.8 to 3.6 times in the 5-FUBM recipients (Table 2).

In normal BM recipients, G-CSF obviously decreased the percentage of mixed spleen colonies on both day 8 and day 12 observations. Erythroid colonies showed a tendency to increase following G-CSF administration (Table 3). As for the spleen colonies of the second recipients, almost all colonies were of the pure erythroid-type irrespective of G-CSF treatment or nontreatment. When 5-FU–treated mice were the first BMT donors, the results were almost the same, including the decrease of mixed spleen colonies in response to G-CSF treatment (data not shown). The exclusive presence of erythroid-type spleen colonies in second recipients is compatible with the observation that spleen colonies on day 12 were lower in numbers than on day 8 in the second recipients, because erythroid-type spleen colonies are known to appear transiently around day 8.
Table 2. Number of Spleen Colonies per Spleen in the First and Second Recipient Mice. Estimated Total Number of CFU-S per Whole Femoral BM in the First Recipient Mice on Day 12 After the First BMT

<table>
<thead>
<tr>
<th></th>
<th>Normal BM Recipients</th>
<th>S-FUBM Recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control G-CSF Treatment</td>
<td>Control G-CSF Treatment</td>
</tr>
<tr>
<td>Spleen colonies* of first recipients of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>15.7 ± 0.9</td>
<td>15.7 ± 0.3</td>
</tr>
<tr>
<td>Day 12</td>
<td>13.0 ± 1.3</td>
<td>13.9 ± 0.9</td>
</tr>
<tr>
<td>Spleen colonies* of second recipients of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>12.5 ± 1.7†</td>
<td>32.7 ± 2.4‡</td>
</tr>
<tr>
<td>Day 12</td>
<td>1.7 ± 0.3†</td>
<td>9.6 ± 0.8‡</td>
</tr>
<tr>
<td>Total number of CFU-S in a femur of first recipients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>17.5 ± 4.5#</td>
<td>97.4 ± 12.6#</td>
</tr>
<tr>
<td>Day 12</td>
<td>2.4 ± 0.7#</td>
<td>28.6 ± 3.9#</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± standard deviation.

* Spleen colonies that developed from 1 x 10⁵ BM nucleated cells of donor mice.
† Spleen colonies that developed from 1 x 10⁶ BM nucleated cells of normal BM recipient mice or 2 x 10⁵ cells from S-FUBM recipient mice.
‡ The total number of CFU-S per whole femoral marrow was estimated from the BM NCC of the first recipients on day 12 after BMT and the number of spleen colonies that developed in the second recipients. See MRA[CFU-S] in the text.

Origin of each spleen colony in secondary recipients. To determine whether each spleen colony of secondary recipients is derived from the first donor or the first recipient, we tried to amplify Y-chromosome-specific sequence and, as a positive control,β-actin sequence using PCR.

To check a sensitivity of this system, samples from a male-derived colony and a female-derived colony were mixed in various ratios and subjected to PCR. A 10% male and 90% female sample showed a faint Y-chromosome-specific band compared with a 100% male sample. In a 1% male and 99% female sample, no Y-chromosome-specific band was visible, as well as in a 100% female sample. For a control study, mixture of male and female BM cells (ratio is 1:1) were transplanted to female mice and each of their spleen colonies was analyzed. Y-chromosome-specific sequence was amplified in half of the colonies, but not in others, as expected (Fig 3A).

For this sex-discriminating experiment, male mice were used for the first BMT donors and female mice were used for the first and second recipients. For each set of experimental conditions, 10 to 20 spleen colonies obtained from two to six second recipients were analyzed. As a result, the Y-chromosome-specific sequence was amplified in each of 85% to 93% of spleen colonies in secondary recipients of normal BM. No significant difference was observed between G-CSF treatment and nontreatment groups (Fig 3B). These results indicate that most of the spleen colonies were derived from BM cells of the first donors. It is compatible with our presumption that G-CSF increases pre-CFU-S cells.

DISCUSSION

We investigated the effect of G-CSF on hematopoiesis in mice following BMT. G-CSF expanded the pool of myeloid cells in the BM. It increased the rate of small GM colonies, indicating that differentiation of myeloid-committed cells was induced by G-CSF treatment. The time-course of the effect of G-CSF on erythroid cells is of great interest. G-CSF treatment decreased the number of erythroid cells in the BM on day 8 after BMT, which is compatible with early erythroid suppression by G-CSF. In contrast, more
erythroid cells were observed in G-CSF–treated mice on day 12 than in control mice, probably because G-CSF increased the number of erythroid progenitors. The lack of a significant WBC increase in response to G-CSF is thought to be due to the G-CSF treatment being too soon and for too short a time after BMT.

G-CSF treatment soon after BMT increased the number of pre–CFU-S, but not the number of recipient spleen colonies. On the other hand, Tamura et al have reported that spleen colonies were increased by the G-CSF treatment of donors immediately before transplantation. The mechanism of these effects of G-CSF on primitive stem cells is uncertain. However, by comparing these observations, we can suggest that G-CSF increases the number of primitive stem cells such as CFU-S and pre–CFU-S either when it is administered to mice having normal hematopoietic tissues or after repopulation of the hematopoietic tissues. G-CSF treatment soon after BMT caused repopulation of myeloid cells by direct stimulation, but could not increase CFU-S, probably due to a lack of repopulated and functioning hematopoietic tissues. After a certain period of time the at least partly repopulated hematopoietic tissues (stimulated by early G-CSF administration) were assumed to increase the progenitors noticed as pre–CFU-S at the time of the first BMT. Thus, G-CSF appears to act on primitive stem cell proliferation through indirect mechanisms.

In our experiments, the increase rate of MRA(CFU-S) in response to G-CSF treatment was higher in normal BM recipients than in 5-FUBM recipients. When repeated BMT was performed, the increase rate of GM-CFC and myeloid cells in the femoral BM in response to G-CSF showed the same tendency on day 12. These observations also suggest repopulated hematopoietic tissues (probably the myeloid fraction) are necessary to allow an increase of pre–CFU-S by G-CSF treatment.

G-CSF decreased mixed spleen colonies and increased erythroid colonies (and granulocytic/monocytic colonies on day 12) without increasing the total number of spleen colonies in recipients of the first BMT. The mechanisms causing these effects are unknown. It is generally assumed that primitive stem cells proliferate and differentiate simultaneously. If G-CSF shortens the time taken for the cell cycle to be completed, the rate of committed progenitors would increase through an increase in the number of cell cycles in a given time period. However, G-CSF did not have a proliferative effect on spleen colonies in the first BMT recipients, so this mechanism appears to be unlikely. Another possible mechanism is the earlier commitment of multipotential stem cells, but no change of the time taken for the cell cycle, in response to G-CSF. Earlier commitment is compatible with both a higher rate of committed cells and no proliferation of CFU-S in response to G-CSF.

We developed a method using PCR to detect the origin of BM cells. Expected sizes of amplified PCR products are 292 and 220 base pairs for Y-chromosome–specific sequence and β-actin sequence, respectively. Numerals at the right side show the sizes (base pairs) of DNA markers and their positions. The recipient was a lethally irradiated female mouse in each lane. (A) The donor was a normal male mouse in lane 1 and normal female in lane 4. In lanes 2 and 3, mixture of samples from a male-derived spleen colony and female-derived colony was subjected to PCR (male: female is 10:90 in lane 2 and 1:99 in lane 3). In lanes 5 through 10, a mixture of male and female BM cells (ratio is 1:1) was transplanted. (B) The donor was the first female recipient on day 12 after BMT from the nontreated male mouse. In lanes 1 through 5, first recipients were treated with G-CSF. In lanes 6 through 10, they were not treated with G-CSF.
of each spleen colony. It may be inevitable, when taking colony samples, to also pick up a little surrounding tissue, including donor-derived male cells. However, in our method discrimination of 50% female-derived spleen colonies was successfully performed in a control study in which mixture of male and female BM cells were transplanted. This finding is compatible with a fact that sensitivity for detecting Y-chromosome-specific sequence is not very high, as shown in Fig 3A. This method is useful to demonstrate whether each CFU-S contained in the marrow of first recipients is derived from the endogenous marrow or from the transplanted marrow. Conventional pre-CFU-S assay by sequential BMT was based on a hypothesis that, in the first BMT recipient, CFU-S is derived from the transplanted marrow. We directly showed it is true. In addition, increased numbers of CFU-S by G-CSF treatment were confirmed to originate mostly from the transplanted marrow. We guess that immature fractions are rarely increased by G-CSF in pre-CFU-S. But, in most experimental conditions, 7% to 15% of CFU-S originated from the endogenous marrow. We guess that immature fractions of CFU-S derived from the endogenous marrow, which is relatively radiation-resistant, survived.

The timing of the administration of G-CSF to BMT patients is still controversial. Our experimental results suggest that it is reasonable to start G-CSF immediately after BMT. Although G-CSF cannot increase the number of neutrophils in the peripheral blood at that time, it will increase myeloid-committed cells in the hypocellular BM and expand the compartment of primitive stem cells. Clinical studies are necessary to confirm this hypothesis.

ACKNOWLEDGMENT

We would like to thank Dr H. Toyoshima, Dr R. Sakai, Dr T. Kaneko, and Dr K. Hagiwara for useful discussions; S. Kurokawa for her excellent technical assistance; and M. Yoshida for preparation of the manuscript.

REFERENCES

Stimulatory effects of granulocyte colony-stimulating factor on colony-forming units-spleen (CFU-S) differentiation and pre-CFU-S proliferation in mice

T Tanaka, T Suda, J Suda, T Inoue, Y Hirabayashi, H Hirai, F Takaku and Y Miura